EXHIBIT G

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N-nitrosodiethylamine genotoxicity evaluation: a cytochrome P450 induction study in rat hepatocytes

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ABSTRACT. In rats, *N*-nitrosodiethylamine (NDEA) induces tumors mainly in the liver. This could be because various enzymes are responsible for the metabolic activation of NDEA, besides the hepatic NDEA metabolizing enzyme, CYP2E1. We examined NDEA genotoxicity and cytotoxicity in primary cultures of female rat hepatocytes; we also looked at how it affected CYP mRNA expression. Single incubation with 0.9% NaCl resulted in a mean of 0.2% apoptotic cells, which doubled with 105 µg NDEA/mL. The frequency of necrosis with NDEA treatment was also doubled. Besides the cytotoxic effects, there was also a 4-fold decrease in mitotic index and a 3-fold decrease in the percentage of cells with micronuclei. A significant increase in micronucleus cells when hepatocytes were incubated with 2.1 µg NDEA/mL suggests that DNA repair was inactive. The chromosomal aberration evaluation revealed a discrete dose-response curve. Treatment with NDEA

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induced increases in CYP mRNA: CYP2B2 (1.8 times) and CYP2E1 (1.6 times) with non-cytotoxic NDEA concentrations (0.21-21 µg/mL). CYP2B1 mRNA levels decreased at 0.21 µg NDEA/mL (2.5-fold), while CYP4A3 mRNA decreased 1.3-fold. NDEA treatment at 2.1 µg/mL induced a 1.9-fold increase in CYP3A1 mRNA. Understanding the cumulative effects in target cells during precarcinogenesis is crucial to understanding the mode of action of potential carcinogens and in order to develop comprehensive chemical toxicity profiles.

Key words: Hepatocytes; NDEA; CYP; Genotoxicity; Cytotoxicity; Clastogenicity

INTRODUCTION

Nitrosamines are a versatile group of carcinogens that produce tumors in many species of animals and in a variety of organs. They require metabolic activation through P450-catalyzed α-hydroxylation, generating unstable metabolites that will alkylate the DNA at the site of activation, inducing tumors (Ribeiro Pinto, 2000).

During the last few years, there has been great interest in developing rapid and simple tests for carcinogens using prokaryotes (Aiub et al., 2003, 2004a,b, 2006, 2009) to identify the effects of exposure to *N*-nitrosodiethylamine (NDEA) on induced DNA damage; eukaryotes have also been used for such studies (Lazarová et al., 2006; Khader et al., 2007, 2009). Hepatocytes, being the cells that express the highest CYP levels, are the most suitable model to investigate CYP induction and their relationship with drug metabolism.

In rats, a commonly used experimental model for carcinogenesis, NDEA induces tumors mainly in the liver. The hypothesis is that various enzymes are responsible for the metabolic activation of NDEA, besides the hepatic NDEA-metabolizing enzyme, CYP2E1 (Ribeiro Pinto and Swann, 1997; Ribeiro Pinto, 2000).

We examined NDEA genotoxicity (micronuclei and mitotic index) and cytotoxicity (survival, apoptosis and necrosis rates) in primary cultures of female rat hepatocytes and how NDEA affects CYP mRNA expression.

MATERIAL AND METHODS

Animal model

This study was conducted in compliance with the NRC "Guide for the Care and Use of Laboratory Animals". Female albino Fischer 344 rats (F-344/DuCrl) from Charles River Laboratories (Germany), 6 to 8 weeks old, were used for the experiments on a 12-h light-dark cycle and supplied with standard pelleted rat diet and water *ad libitum*. We analyzed liver cells from three female Fischer 344 rats for each NDEA concentration.

Hepatocyte isolation and culture

Hepatocytes were prepared with the two-step collagenase perfusion method (Khader

et al., 2007). Rats were anesthetized with sodium pentobarbital (200 mg/kg), and following hepatic portal vein cannulation, livers were perfused with 200 mL solution A (142 mM NaCl, 6.7 mM KCl, 10 mM HEPES, pH 7.4) for 15 min at 15 mL/min. Livers were subsequently perfused with 200 mL solution C (9:1 of solution A and 5.7 mM $CaCl_2$, pH 7.4) containing 0.5 mg collagenase/mL (Collagenase Sigma IV, 125 CDU/mg, CAS 9001-12-1) for 20 min at 10 mL/min. Perfused livers were excised and dispersed in 50 mL solution A and shaken in a closed, sterile container at 37°C for 10 min. The hepatocyte preparation was filtered through a 180-µm nylon filter and centrifuged at 500 rpm, for 10 min, at 4°C. The wash was repeated once and the cells resuspended in 1.8 mM MEM Eagle Ca^{2+} (Gibco), supplemented with 26.2 mM $NaHCO_3$, 1 mM pyruvate, 0.2 mM aspartic acid, and 0.2 mM L-serine. Hepatocytes were counted by hemacytometry, and 2-5 x 10^5 cells/mL were added to 60- and 90-mm collagen-coated dishes. Hepatocytes were allowed to attach for 3 h. Viability was approximately 85-90%. After attachment, the medium was removed and replaced with fresh 1.8 mM MEM Eagle Ca^{2+}.

Incubation of cells

After 16 h, the rat hepatocytes were incubated for 3 h in NDEA concentrations ranging from 0.21 to 105 µg/mL (final concentrations). The cells were washed once with 0.4 mM MEM Eagle and reincubated with 0.4 mM MEM Eagle for 48 h, with EGF (40 ng/mL, final concentration) and 0.1 µM insulin (final concentration). RNA was extracted after 6 h and cytogenetic assays were made after 48 h of NDEA treatment (0.21-105 µg/mL, dissolved in 0.9% NaCl). Cells were treated with 0.5 µM N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as a positive control for cytogenetic assays.

Cytogenetic studies

Cytogenetic studies were performed in triplicate as described by Eckl and Riegler (1997), with some modifications. For the determination of the mitotic index and the number of cells with micronuclei, the 0.4 mM MEM Eagle was replaced with cold methanol-glacial acetic acid (3:1) fixative for 15 min on Petri dishes, which were then rinsed with distilled water for 2 min and air dried. The fixed cells were stained with DAPI (0.2 pg/mL) dissolved in McIlvaine buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 7.0) for 40 min, washed with McIlvaine buffer for 2 min, briefly rinsed with distilled water and mounted in glycerol. To determine the mitotic index and the number of cells with micronuclei, 1000 cells per Petri dish (2000 cells per animal/group concentration) were analyzed under a fluorescence microscope (Reichert Univar). Micronucleus results are reported as the percentage of cells containing micronuclei in 2000 cells/group concentration analyzed. Cells with bright glowing and homogenous nuclei were considered as having normal phenotype morphology. Apoptotic nuclei were identified by the condensed chromatin at the periphery of the nuclear membrane or by a fragmented nuclear body morphology. Necrotic cells presented chromatin forms with irregularly shaped aggregates and a pyknotic nucleus (shrunken and darkly stained), and the cell membrane disrupted, with cellular debris spilled into the extracellular milieu. Again, 2000 cells were counted and the percentage of apoptotic and necrotic cells determined.

For chromosomal aberrations, we added colcemid (0.4 µg/mL) to the dishes (triplicate/animal/NDEA group concentration) and incubated the cells for a further 3 h. The medium

was replaced by 2 mL collagenase solution (0.5 mg/mL) for 10 min in order to detach the cells. The cells were then collected and centrifuged. The collagenase solution was replaced with hypotonic KCl solution (0.01 M) for 10 min and fixed in cold methanol-glacial acetic acid (3:1), overnight. Preparations were made by dropping the cell suspension in fixative on glass slides. Five slides/animal/NDEA group concentration were made. The slides were stained using Hoechst 33258 (4.5 µg/mL) for 15 min, rinsed with distilled water, mounted with a coverglass in PBS, pH 7.0, and exposed for 1 h to a 40-W blacklight lamp (Philips TLD 36W08) at 50°C. The coverslips were removed and the slides were stained with 5% Giemsa solution. Chromosomal aberrations per animal were scored using 50 well-spread metaphases. The number of aberrations was given per diploid cell, i.e., 42 chromosomes. Metaphases were scored for chromosome-type aberrations, such as chromosome deletions, dicentric and ring chromosomes.

RNA isolation

Total RNA was isolated with TRIzol (Invitrogen, Germany) and treated with DNase I (0.5 U) (Invitrogen), according to manufacturer instructions, and dissolved in DEPC water. The concentration of the isolated total RNA was spectrophotometrically determined at 260 nm and diluted in DEPC water at 2 µg/µL. Aliquots of RNA were analyzed by agarose/formadehyde gel electrophoresis to check RNA integrity and stored at -20°C until further use.

Development of primers for quantitative real-time PCR

Coding sequences for the genes (Table 1) were obtained from GenBank (http://www.ncbi.nml.nih.gov/GenBank). Target regions within the coding sequences were determined through nucleotide sequence alignment comparisons of target within multiple member gene families using Vector NTI (Informax, Inc., Bethesda, MD). A subfamily-specific region for each CYP was selected as the site of hybridization for either the 5' or 3'CYP PCR primer, and then complementary PCR oligos were screened on the basis of i) similar melting temperatures, ii) similar oligo length, and iii) the production of a PCR amplicon with greater than 50% GC content. All primers were submitted to the National Center for Biotechnological Information for nucleotide comparison using the basic logarithmic alignment search tool (BLASTn) to ensure specificity.

cDNA synthesis

First-strand cDNA synthesis was performed on the remaining DNase-treated total RNA, which was reverse-transcribed using oligo primers (0.3 ng/µL) and Superscript™ III bulk mix (Invitrogen), according to manufacturer instructions. In addition, duplicate no-template control samples were run under identical conditions.

Real-time qRT-PCR analysis

Quantitative RT-PCRs were performed using the reagent mix and protocol contained in the BioRad iCycler iQ Real-Time Detection System (BioRad). Reactions were run in duplicate, in a volume of 10 µL, containing 5 µL cDNA diluted 1:20 in DEPC water, 4.4 µL iQ

SYBR Green Supermix, and 0.6 µL of the primer mix (forward and reverse primers) at 3 pmol/µL (Table 1). The cycle was programmed for 95°C, 3 min; 40X (95°C for 1 min, 65°C for 1 min and 72°C for 1 min); 95°C for 1 min, and 55°C for 1 min. For the melting curve analysis, we used a gradient from 55° to 95°C. Real-time PCR data were collected and analyzed on a Rotor-Gene 3000 (Corbett Research). The products were checked by agarose gel electrophoresis and by sequencing. CYP2A2 was used as a negative control for CYP expression.

The endpoint used in the real-time PCR quantification, cycle threshold (CT) value, was defined as the PCR cycle number that crosses an arbitrarily placed signal threshold. Mean CT values from duplicate PCRs were normalized to average CT values for housekeeping gene from the same cDNA preparations (ΔCT). The ratio of expression of each CYP gene induced *vs* expression of each CYP gene spontaneously induced was calculated as ΔΔCT. The fold induction of each CYP gene was calculated as $2^{-(\Delta\Delta CT)}$, as recommended by Perkin-Elmer. Values are reported as means of triplicate analyses. The amount of each gene target in different groups was normalized to an endogenous control (18S ribosomal RNA). GADPH was used in order to compare with the best endogenous control for hepatocytes in culture.

Table 1. Primer sequences used for real-time quantitative PCR.

Gene/location	Tm (°C)	Primer	Sequence	Amplicon (bp)
18S RNA	67.5	Forward	AGTCCCTGCCCTTTGTACACACCGC	152
(V01270)	69.5	Reverse	ACCATCCAATCGGTAGTAGCGACGGG	
GAPDH	68.1	Forward	TGCCTCTCAGACAATGCCTGGATCCCTAA	159
	70.5	Reverse	GGCCAGCCCTTACTTATACTTGGATGTTCCTTCTCTTA	
CYP2A1	68.1	Forward	TCAATCCTCACTGGCCACTATGCTGGACA	90
(J04187)	69.5	Reverse	CAGAGGGACACCAAGAGCATGACGCTC	
CYP2A2	68.4	Forward	AGACACAGTTAGCTAGGATTGACACATTGGACTCTAT	188
(M34392)	68.4	Reverse	ATAGCTTTGGATACAGATCTGGACTCTTATGGTCTAAG	
CYP2B1	68	Forward	CAGCCAGGTGTTTGAGTTCTTCTCTGGG	122
(AJ320166)	66.4	Reverse	CCCTGTGCTTCTCCACAATATGGCCA	
CYP2B2	66.1	Forward	ACATGTGAACAGAGATTTCATGAGTACACATCTCAT	148
(J00720)	68.3	Reverse	TGTAGACATAGCACTGAGACCATATACAGAGTCCAT	
CYP2E1	66.8	Forward	CTCCTCGTCATATCCATCTGGAAGAAGATCT	127
(J02627)	63.7	Reverse	TGGTGAAAGACTTGGGGATATCCTTCAA	
CYP3A1	63.9	Forward	GTTTATGGAAATTCGATGTGGAGTGCCAT	153
(M10161)	64.4	Reverse	CCCGCCGGTTTGTGAAGACAGAAA	
CYP4A3	66.8	Forward	CACAGATGGAGTGATCAAAATGAGGAAGGC	148
(M33936)	68	Reverse	CAGGTCCTCATCAGACAAGCTCTTCCCA	

Tm = melting temperature.

RESULTS AND DISCUSSION

Cytogenetic studies

DNA fragmentation is a marker for genotoxic effects, confirmed by the micronucleus assay and chromosomal aberration counts. Apoptosis rate and viability were also assessed in order to inform about induced cytotoxicity.

Cell viability was consistently high (mean 97.2% at the highest NDEA concentration); therefore, cytotoxic effects could be compared with the control group. Single incubation with 0.9% NaCl used as a negative control resulted in median fractions of apoptotic cells of 0.2%, which doubled with NDEA at 105 µg/mL. Necrosis at the highest NDEA dose was also double that of the control (Table 2).

Table 2. Cytotoxicity evaluation after *N*-nitrosodiethylamine (NDEA) treatment.

Assay conditions	% Survival	% Necrosis	% Apoptosis
0.9% NaCl	98.7 ± 0.3	1.1 ± 0.4	0.2 ± 0.1
0.21 µg NDEA/mL	98.2 ± 0.4	1.6 ± 0.3	0.2 ± 0.1
2.1 µg NDEA/mL	97.4 ± 0.5	2.4 ± 0.5*	0.2 ± 0.1
21 µg NDEA/mL	97.2 ± 0.7	2.4 ± 0.6*	0.4 ± 0.2
105 µg NDEA/mL	97.2 ± 1.0	2.5 ± 1.0	0.4 ± 0.1*
MNNG (0.5 µM)	99.6 ± 1.2	0.1 ± 0.03	0.31 ± 0.12

Data are reported as means ± SEM of three female Fischer 344 rat livers; 2000 cells were analyzed per animal/NDEA group concentration. MNNG = N-methyl-N'-nitro-N-nitrosoguanidine (positive control). Statistical analysis was used to compare the NDEA cytotoxic effects compared to the control group (0.9% NaCl). *P < 0.05.

Beside the cytotoxic effects at the highest NDEA concentration (105 µg/mL), a decrease in mitotic index (four times compared with the control) and percentage of cells with micronuclei (three times compared with the control) was observed. But a significant increase in micronucleus cells was observed when hepatocytes were incubated in 2.1 µg NDEA/mL (Table 3).

Table 3. *N*-nitrosodiethylamine (NDEA) genotoxicity.

Assay conditions	Mitotic index (%)	Micronucleus cells (%)
0.9% NaCl	4.2 ± 1.1	9.3 ± 2.3
0.21 µg NDEA/mL	4.1 ± 0.1	11.7 ± 4.2
2.1 µg NDEA/mL	4.2 ± 0.4	17.3 ± 2.3*
21 µg NDE/mL	2.3 ± 0.6	6.0 ± 2.6
105 µg NDEA/mL	1.1 ± 0.6*	3.3 ± 0.6
MNNG (0.5 µM)	1.8 ± 3.0	18 ± 2.7

Data are reported as means ± SEM of three female Fischer 344 rat livers; 2000 cells were analyzed per animal/NDEA group concentration. MNNG = N-methyl-N'-nitro-N-nitrosoguanidine (positive control). Statistical analysis was used to compare the NDEA cytotoxic effects compared to the control group (0.9% NaCl). *P < 0.05.

The evaluation of chromosomal aberrations (Table 4) revealed no significant differences in DNA damage induced by NDEA, although a dose-response curve was observed.

Table 4. Number of chromosomal aberrations per cell in female Fischer 344 rat hepatocytes after *N*-nitrosodiethylamine (NDEA) treatment.

Assay conditions	Chromosome damage			Total damage ± SD
	Deletions/breakage	Dicentrics	Rings	
NaCl 0.9%	1.23	0.36	0.64	2.23 ± 1.30
0.21 µg NDEA/mL	1.42	1.26	0.47	3.15 ± 1.70
2.1 µg NDEA/mL	0.94	2.37	0.63	3.94 ± 1.90
21 µg NDE /mL	1.47	3.53	0.41	5.41 ± 0.21*
105 µg NDEA/mL	1.17	2.50	0.30	4.00 ± 0.01
MNNG (0.5 µM)	9.34	2.06	3.27	14.67 ± 3.45

MNNG = N-methyl-N'-nitro-N-nitrosoguanidine (positive control). Statistical analysis was used to compare the NDEA cytotoxic effects compared to the control group (0.9% NaCl). *P < 0.05.

CYP expression

The CYP mRNA data are reported as fold induction of the house keeping gene (18S), under different NDEA concentrations, measured by real-time PCR. The NDEA treatment induced an alteration in CYP mRNA, with an increase in CYP2B2 (1.8 times) and CYP2E1 (1.6 times) expression, with non-cytotoxic NDEA concentrations (0.21-21 µg/mL). In the case of CYP2B1, an increase in mRNA levels was detected only at 0.21 µg/mL (2.5 times) and 1.3 times for CYP4A3. NDEA treatment induced an increase in CYP3A1 mRNA - 1.9 times, at 2.1 µg/mL (Table 5).

Table 5. Fold induction (measured by real-time PCR) of 18S rat CYP genes following administration of *N*-nitrosodiethylamine (NDEA).

Gene	Assay conditions	Mean fold induction
CYP2A1	0.9% NaCl	1.38 ± 0.09
	0.21 µg NDEA/mL	1.40 ± 0.20
	2.1 µg NDEA/mL	1.41 ± 0.14
	21 µg NDEA/mL	0.81 ± 0.24
	105 µg NDEA/mL	0.79 ± 0.54
CYP2B1	NaCl 0.9%	0.84 ± 0.13
	0.21 µg NDEA/mL	2.14 ± 0.03*
	2.1 µg NDEA/mL	0.98 ± 0.33
	21 µg NDEA/mL	0.98 ± 0.30
	105 µg NDEA/mL	0.60 ± 0.24
CYP2B2	NaCl 0.9%	0.85 ± 0.06
	0.21 µg NDEA/mL	2.40 ± 0.23*
	2.1 µg NDEA/mL	1.29 ± 0.15*
	21 µg NDEA/mL	1.58 ± 0.24*
	105 µg NDEA/mL	0.49 ± 0.12*
CYP2E1	NaCl 0.9%	0.72 ± 0.23
	0.21 µg NDEA/mL	1.86 ± 0.15*
	2.1 µg NDEA/mL	1.50 ± 0.20*
	21 µg NDEA/mL	1.14 ± 0.10*
	105 µg NDEA/mL	0.77 ± 0.22
CYP3A1	NaCl 0.9%	1.05 ± 0.15
	0.21 µg NDEA/mL	0.83 ± 0.28
	2.1 µg NDEA/mL	1.95 ± 0.48*
	21 µg NDEA/mL	0.98 ± 0.23
	105 µg NDEA/mL	0.75 ± 0.17
CYP4A3	NaCl 0.9%	0.67 ± 0.20
	0.21 µg NDEA/mL	1.06 ± 0.14*
	2.1 µg NDEA/mL	0.91 ± 0.37
	21 µg NDEA/mL	0.89 ± 0.23
	105 µg NDEA/mL	0.91 ± 0.22

Data are reported as means ± SEM of 2000 cells from each of three female Fischer 344 rat livers. *Significant difference ($P < 0.05$) between the NDEA treatment and 0.9% NaCl (control).

Discrimination of cumulative effects in target cells during carcinogenesis is crucial to understanding the mode of action of potential carcinogens and to develop comprehensive chemical toxicity profiles. *In vitro* studies have shown that NDEA needs to be processed intracellularly to cause damage (Aiub et al., 2003, 2004a,b, 2006, 2009). Different cytochrome P450-dependent monooxygenases, including CYP2A and CYP2B groups and CYP2E1, are considered to be key enzymes involved in the activation or inactivation of NDEA. The extent

of this biotransformation depends on the tissue specific availability of the necessary P450 isoenzymes (Camus et al., 1993). Metabolic activation can lead to the local formation of DNA adducts, impairment of bases and DNA breaks (Camus et al., 1993; Koskela et al., 1999; Aiub et al., 2003, 2004a,b).

We observed that NDEA provoked a significant increase in micronucleus induction and DNA fragmentation (Tables 3 and 4). The increase in micronucleus induction came close to the mitotic index, suggesting that DNA repair is inactivated. The cells are able to repair the DNA damage since TP53 protein stops the cell cycle for this event. It has been shown that low doses of NDEA result in moderate activation of TP53, without the initiation of an apoptotic reaction, in contrast with a single high-dose exposure, which causes massive activation of proapoptotic mechanisms (Lim, 2003). Our data are in accordance with the hypothesis that the DNA damage induced by NDEA corresponds to an increase in micronuclei due to DNA breakage that could not be repaired, leading to an increase in chromosomal aberrations, and an increase in apoptotic cells.

Microsomal generation of reactive oxygen species (ROS) is one of the most significant causes of liver injury. NADPH-dependent production of ROS by animal liver microsomes has been linked to CYP activation. It has been suggested that NDEA, one of the most important environmental carcinogens, besides being metabolized to reactive electrophiles, causes the generation of ROS, resulting in oxidative stress and cellular injury. NDEA induces CYP2B1 expression, which plays a major role in the activation of long-chain nitrosodialkylamines, such as N-nitrosodibutylamine (Shu and Hollenberg, 1997). The uncoupling of electron transfer and oxygen reduction from monooxygenation by CYP2B1, CYP2B2 and CYP2E1 could result in the release of O_2 and H_2O_2, and consequently an increase in these metabolites can increase DNA damage. A recent study has demonstrated that CYP1A1, CYP3A, and CYP4A are also involved in this step (Liu et al., 2005), and we suggest the same for CYP3A1 and CYP4A3.

Several P450 cytochromes may be involved in these reactions. A prominent phenomenon during hepatocarcinogenesis is alteration of the expression of drug metabolizing enzymes; in this case NDEA, acting as a precarcinogen, induced CYP expression alterations.

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